09/235160

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: MODIFICATION OF LIGNIN SYNTHESIS IN PLANTS

(57) Abstract

The biosynthesis of lignin in plants is regulated by insertion into the plant genome by altering the plant's ability to synthesize the enzyme O-methyl-transferase, an enzyme involved in the lignin biosynthetic pathway. Production of O-methyl-transferase may be enhanced by insertion into the plant genome by transformation of one or more additional copies of the O-methyl-transferase gene or production may be inhibited by insertion of a gene encoding anti-sense mRNA directed against the mRNA encoded by the endogenous O-methyl-transferase gene.

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MODIFICATION OF LIGNIN SYNTHESIS IN PLANTS

This invention relates to the improvement of
plants by the modification of lignin biosynthesis,
particularly, but not exclusively, the improvement
of digestibility of fodder crops.

5   Grassland farmers, and farmers of other
fodder crops, face a difficult decision each year
about when to cut their crops for conservation.
All grass varieties of agricultural importance
suffer from the disadvantage that during the normal
10   increase in dry matter yield with growth, the
digestibility decreases. The farmer, therefore,
has, to compromise between a lower yield of highly
digestible material and a higher yield of less
digestible material. Another limitation is that
15   harvesting at optimum maturity may be prevented by
unfavourable weather. If the decline in
digestibility could be controlled or delayed,
higher yields of highly digestible material could
be obtained and the prevailing weather conditions
20   would not play such a major role in determining the
quality of the harvested crop.
       Digestibility of fodder crops is determined,
among other factors, by the amount and quality of
lignin deposition which has taken place during
25   growth of the plants and the degree of secondary
modification of lignin deposited. Beside cellulose
and other poly- saccharides, lignins are an

essential component of cell wall in tissues like
the sclerenchyma and the xylem of vascular plants.
They play an important role in the conducting
function of the xylem by reducing the permeability

5   of the cell wall to water. They are also
responsible for the rigidity of the cell wall, and,
in woody tissues, they act as a bonding agent
between cells, imparting to the plant a resistance
towards impact, compression and bending. Finally,

10   they are involved in mechanisms of resistance to
pathogens by impeding the penetration or the
propagation of the pathogenic agent.

Lignins are not only important in the
productivity and performance of field crops but are

15   also of great importance in trees for paper making.
Considerable energy and chemical input is required
to loosen, dissolve and remove lignin from the
cellulose fibre which is required for paper making.
In addition to these instances in which lignins

20   present a constraint on the use of crop plants,
lignins are also used as feedstocks for the
preparation of speciality chemicals such as
phenolics which can be used as precursors in
chemical synthesis. Thus lignins and their

25   biological and chemical modification are important.

It is one of the objects of the present
invention to provide a biotechnological procedure
for the modification both lignin content and lignin
composition in plants.

30   Lignins are the product of a dehydrogenative
polymerisation of three primary precursors: the
trans-coniferyl, trans-sinapyl and trans-p-coumaryl
alcohols. The monomers can occur in lignins in
different proportions and with different types of

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links both with each other and with the surrounding
cell wall polysaccharides,thus producing a wide
variety of polymers. These polymers, or "lignin
cores" are always associated covalently with
5   hemicelluloses. Most lignins also contain varying
amounts of aromatic carboxylic acids in ester-like
combinations. Such differences in the structure of
lignins are usually found in plant species.
However, differences in the composition of lignins,
10   and even in the binding to the primary and
secondary cell walls, can also occur in the same
plant, between different tissues of different ages.
The biosynthesis of lignin monomers (monolignols)
is a part of the phenylpropanoid biosynthesis
15   pathway, which is also responsible for the
production of a wide range of compounds including
flavonoid pigments, isoflavonoids, coumarin
phytoalexins and cell division promoting
dehydrodiconiferyl glucosides.
20   Phenylalanine is deaminated to produce
cinnamic acid. This acid is then transformed by
hydroxylation and methylation reactions, thus
producing different acids substituted on the
aromatic ring. The enzyme catalysing the
25   methylation steps is O-methyl transferase (OMT).
O-methyltransferases (S-adenosyl-L-methionine:
O-methyltransferases; EC 2.1.1.6) thus play an
important role in the biosynthesis of monolignols.
By the O-methylation of caffeic acid and
30   5-hydroxyferulic acid, OMTs introduce one and two
methoxy groups in the lignin monomers,
respectively. The resulting two phenolics, ferulic
acid and sinapic acid, respectively, are the
precursors of coniferyl alcohol and sinapyl alcohol

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which are together with coumaryl alcohol substrates for peroxidases (Lewis and Yamamoto, 1990).

The previous methylation reactions are also used in the synthesis of several other phenolic
5 compounds. However, in those cells which are dedicated to the production of lignins such as vascular xylem cells of plants, the OMT plays a crucial role in the production of the phenolic precursors incorporated into the lignin polymer.
10 The cinnamyl alcohols, synthesised in the cytoplasm, are then transported to the cell wall where they are polymerised by peroxidase in the presence of hydrogen peroxide.

When the surface growth of the cell ceases, it
15 is followed by a phase of wall thickening (secondary wall formation). Lignification takes place during this phase. It starts in the cell corners and extends along the middle lamella, through the primary wall and, finally, to the
20 secondary wall. External factors can induce qualitative and quantitative modifications in lignification. The synthesis of new types of lignins, sometimes in tissues which are not normally lignified, may be induced by infection
25 with pathogenic microorganisms. Lignification is stimulated by light, as well as by low calcium levels, by boron, by mechanical stress and by infection.

As a first step in unravelling of lignin
30 biosynthesis at the molecular level, we have undertaken the biochemical characteristics and cloning of O-methyltransferases (OMTs). Previously three different OMTs (OMT I, OMT II, and OMT III) have been purified from tobacco. OMT I used mainly

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caffeic acid and 5-hydroxyferulic acid as a
substrate and is the OMT actively present in
healthy plants. OMT II and OMT III have a broader
substrate specificity and also use catechol as

5   substrate. Upon infection with TMV, an increase in
activity of all three OMTs was shown. Based on
this observation, it has been postulated that the
OMT I is specifically involved in lignification,
whereas OMT II and OMT III have a function in

10   generating a lignin barrier upon infection. The
importance of methylation in monolignol
biosynthesis is well illustrated in brown-rib corn
mutants. These plants exhibit a reduced lignin
content and accumulate 5-hydroxyferulic acid) due

15   to a low O-methyltransferase activity. Thus OMTs
could be potential targets for modulation of
lignification through the use of recombinant DNA
technology.

Thus, plants with a reduced amount of lignin
20   would be more efficiently used as a forage for
cattle. The yield of milk and meat would be
therefore increased. Furthermore, lignin may have
a negative effect on plant growth. Thus, a
reduction of the lignification in crops such as

25   wheat, oilseed rape, sugar beet or maize might
presumably increase the grain yield. Trees with
reduced lignin contents or altered lignin structure
will lead to a reduction in the cost of the paper
as less lignin will have to be removed during the

30   pulping process. On the other hand, novel papers
may be produced due to the purity of cellulose
fibre which could otherwise not be produced.

Reduction of lignification can be achieved by
the application of chemical inhibitors to plants.

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However, a more effective method controlling lignin deposition and structure is the inhibition of expression of the CAD gene using antisense RNA. Antisense RNA technology is an appropriate

5 molecular biology approach to the inhibition of lignification. An antisense RNA is an RNA produced by the transcription of the non-coding DNA strand (nonsense). Thus, antisense RNA has the same sequence as the coding DNA strand and is

10 complementary to the mRNA product of a specific gene.

As is well known, a cell manufactures protein by transcribing the DNA of the gene for that protein to produce RNA, which is then processed

15 (e.g. by the removal of introns) into messenger RNA and finally translated by ribosomes into protein. This process may be inhibited by the presence in the cell of "antisense RNA". Therefore, as used herein, the term "antisense RNA" means an RNA

20 sequence which is complementary to a sequence of bases in a mRNA: complementary in the sense that each base (or a majority of bases) in the antisense sequence (read in the 3' to 5' sense) is capable of pairing with the corresponding base (G with C, A

25 with U) in the mRNA sequence read in the 5' to 3' sense. It is believed that this inhibition takes place by formation of a complex between the two complementary strands of RNA, preventing the formation of protein. How this works is uncertain:

30 the complex may interfere with further trans- cription, processing, transport or translation, or lead to degradation of the mRNA, or have more than one of these effects. Such antisense RNA may be produced in the cell by transformation with an

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appropriate DNA construct arranged to transcribe
backwards part of the coding strand (as opposed to
the template strand) of the relevant gene (or of a
DNA sequence showing substantial homology
5   therewith).

The use of this technology to down-regulate
the expression of specific plant genes has been
described, for example in European Patent
Publication No 271988 to ICI. Reduction of gene
10 expression has led to a change in the phenotype of
the plant: either at the level of gross visible
phenotypic difference e.g. lack of lycopene
synthesis in the fruit of tomato leading to the
production of yellow rather than red fruit or at a
15 more subtle biochemical level e.g. change in the
amount of polygalacturonase and reduction in
depolymerisation of pectins during tomato fruit
ripening (Smith et al, Nature, 334, 724-726, 1988;
Smith et al, Plant Mol Biol 14, 369-380, 1990).
20 Thus antisense RNA has been proven to be useful in
achieving down-regulation of gene expression in
plants.

An object of the present invention is to
provide plants having an altered ability to
25 synthesise lignin.

According to the present invention there is
provided the DNA insert contained in the clones
pPLC4 and pTOMTI and variants thereof such as are
permitted by the degeneracy of the genetic code or
30 the functional equivalents thereof.

In addition, the present invention provides a
recombinant DNA construct containing the said DNA
under control of a transcriptional control sequence
operative in plants, so that the construct can

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generate mRNA in plant cells.

For the down-regulation of lignin synthesis the aforesaid DNA is in antisense orientation.

For the amplification of lignin biosynthesis
5   the aforesaid DNA is in sense orientation thus to provide one or more additional copies of the said DNA in the plant genome.

Thus, in a further aspect, the present invention provides DNA constructs comprising a
10   transcriptional initiation region operative in plants positioned for transcription of a DNA sequence encoding RNA complementary to a substantial run of bases showing substantial homology to an mRNA encoding the protein produced
15   by the gene in pPLC4 pTOMT1.

The invention further provides plant cells, and plants derived therefrom having stably incorporated in their genomes the aforesaid DNA in sense or antisense orientation, and fruit and seeds
20   of such plants. The present invention is principally concerned with the suppression of lignin formation and, that being so, the inserted gene will be in antisense orientation, but there are instances where over-production of lignin may
25   have an advantageous effect, for example to improve plant stalk strength and resistance to diseases, and the present invention provides means for achieving amplification of the lignin biosynthetic ability of plants.

30   Thus the invention relates generally to the regulation of the plant's lignin biosynthetic pathway, in which OMT plays a dominant role, in order that the production of OMT, and hence the production and composition of lignin is altered by

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insertion of the OMT gene, or a portion thereof
(usually of 50 or more bases), in antisense
orientation so that the amount of OMT for
catalysing lignin synthesis is reduced.

5   The constructs of the invention may be
inserted into plants to regulate the production of
the CAD enzyme. Depending on the nature of the
construct, the production of the protein may be
increased, or reduced, either throughout or at
10   particular stages in the life of the plant. It is
also possible to target the expression of the gene
to a specific cell types of the plant, such as the
epidermis, the xylem, the roots etc.

The plants to which the present invention can
15   be applied include commercially important food and
forage plants, such as alfalfa, maize, oil seed
rape, forage grasses and sunflower, and but also
tree crops such as eucalyptus, pine species and
poplar.

20   DNA constructs according to the invention
preferably comprise a sequence of at least 50 bases
which is homologous to the DNA of the insert in
pPLC4 or pOMT1.A and pOMT1.B There is no
theoretical upper limit to the base sequence - it
25   may be as long as the relevant mRNA produced by the
cell - but for convenience it will generally be
found suitable to use sequences between 100 and
1000 bases in length. The preparation of such
constructs is described in more detail below.

30   The preferred source of antisense RNA for use
in the present invention is DNA derived from the
clone pPLC4 or pOMT1.A and pOMT1.B The required
DNA encoding antisense RNA can be obtained in
several ways: by cutting an appropriate sequence of

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DNA from pPLC4 or pOMT1.A or pOMT1.B (or any other
source of the OMT gene); by synthesising a DNA
fragment using synthetic oligonucleotides which are
annealed and then ligated together in such a way as
5   to give suitable restriction sites at each end; by
using synthetic oligonucleotides in a polymerase
chain reaction (PCR) to generate the required
fragment with suitable restriction sites at each
end. The DNA is then cloned into a vector
10   containing upstream promoter and downstream
terminator sequences, the cloning being carried out
so that the DNA sequence is inverted with respect
to its orientation to the promoter in the strand
from which it was cut. In the new vector, the
15   strand that was formerly the template strand
becomes the coding strand, and vice versa. The new
vector will thus encode RNA in a base sequence
which is complementary to the sequence of pPLC4 and
pTOMT1 mRNAs. Thus the two RNA strands are
20   complementary not only in their base sequence but
also in their orientations (5' to 3').

As source of the DNA base sequence for
transcription, it is convenient to use a cDNA clone
such as pPLC4. The base sequence of pPLC4 is shown
25   in Figure 1 and the base sequences of pOMT1.A and
pOMT1.B is shown in Figure 2.

The clone pPLC4 has been deposited at the
National Collections of Industrial and Marine
Bacteria, PO Box 31, of 23 St Machar Drive,
30   Aberdeen AB2 1RY, Scotland, as a plasmid in E.coli,
strain 'sure', under the reference NCIB 40436 on
August 22, 1991.

The clone pTOMT1.A has been deposited at the
National Collections of Industrial and Marine

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Bacteria, PO Box 31, of 23 St Machar Drive,
Aberdeen AB2 1RY, Scotland, as a plasmid in E.coli,
strain DH5α, under the reference NCIB 40439 on
September 4, 1991.

5   The clone pTOMT1.B has been deposited at the
National Collections of Industrial and Marine
Bacteria, PO Box 31, of 23 St Machar Drive,
Aberdeen AB2 1RY, Scotland, as a plasmid in E.coli,
strain DH5α, under the reference NCIB 40440 on
10   September 4, 1991.

         A source of DNA for the base sequence for
transcription is the promoter of the OMT gene
itself or other genes involved in lignification
such as the promoter of the phenyl alanine ammonia
15   lyase gene or its modified version which permits
expression in xylem tissue, or the s-Adenosyl
methionine synthase gene or the promoter of the
extensin gene. Such a gene may differ from the
cDNA of pPLC4 and pOMT1A or pOMT1.B in that introns
20   may be present. The introns are not transcribed
into mRNA (or, if so transcribed, are subsequently
cut out). When using such a gene as the source of
the base sequence for transcription it is possible
to use either intron or exon regions.

25   A further way of obtaining a suitable DNA
base sequence for transcription is to synthesise it
ab initio from the appropriate bases, for example
using Figure 1 and Figure 2 as a guide. Recombinant
DNA and vectors according to the present invention
30   may be made as follows. A suitable vector
containing the desired base sequence for
transcription (for example pPLC4 and pOMT1.A and
pOMT1.B) is treated with restriction enzymes to out
cut the sequence. The DNA strand so obtained is

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cloned (in reverse orientation) into a second
vector containing the desired promoter sequence
(for example cauliflower mosaic virus 35S RNA
promoter or the bean PAL promoter, Bevan et al,
5   EMBO J.8, 1899-1906 1988) and the desired
terminator sequence (for example the 3' of the
Agrobacterium tumefaciens nopaline synthase gene.

In this invention we may use both constitutive
promoters (such as cauliflower mosaic virus 35S
10   RNA) and inducible or developmentally regulated
promoters (such as the PAL gene promoter) as
circumstances require. Use of a constitutive
promoter will tend to affect functions in all parts
of the plant: while by using a tissue specific
15   promoter, functions may be controlled more
selectively. The use of a tissue-specific promoter,
has the advantage that the antisense or sense RNA
is only produced in the tissue in which its action
is required.

20   Vectors according to the invention may be
used to transform plants as desired, to make plants
according to the invention. Dicotyledonous plants,
such as alfalfa, oil seed rape etc, may be
transformed by Agrobacterium Ti plasmid technology,
25   for example as described by Bevan (1984) Nucleic
Acid Research, 12, 8711-8721. Such transformed
plants may be replicated sexually, or by cell or
tissue culture.

Poplar and aspen transformation using
30   Agrobacterium tumefaciens can be performed as
described by De Block [Plant Physiol. (1990)
93:1110-1116]. Stem internode pieces are used as a
tissue source for incubation with an Agrobacterium
tumefaciens strain (C58CRif^R(pMP90.) harbouring

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chimeric marker genes (bar/neo) on its non-oncogenic T-DNA. For the aspen clone (Populus alba x P. tremula; clone 357, Afocel) and the poplar clone (Populus trichocarpa x P. deltoides; clone

5   064, Afocel), transgenic shoots were obtained 3 months and 6 months after incubation, respectively.

The degree of production of RNA in the plant cells can be controlled by suitable choice of promoter sequences, or by selecting the number of

10   copies, or the site of integration, of the DNA sequences according to the invention that are introduced into the plant genome. In this way it may be possible to modify lignification to a greater or lesser extent.

15   The constructs of our invention may be used to transform cells of both monocotyledonous and dicotyledonous plants in various ways known to the art. In many cases such plant cells (particularly when they are cells of dicotyledonous plants) may

20   be cultured to regenerate whole plants which subsequently reproduce to give successive generations of genetically modified plants. Examples of genetically modified plants according to the present invention include, alfalfa, oil seed

25   rape, sunflower, sorghum, maize, festuca,and trees such as eucalyptus, poplar, and pine.

In the present invention, we use antisense RNA in order to determine the phenotype of transgenic plants which show modified, that is increased or

30   reduced, expression of pPLC4 or pTOMT1 by the use of antisense and sense expression vectors.

The invention will now be described further with reference to the accompanying drawings, in which:

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Figure 1 shows the complete nucleotide sequence and deduced amino acid sequence from pPLC4.

Figure 2 shows the combined nucleotide
5   sequence and deduced amino acid sequence of pOMT1.A and pOMT1.B.

Figure 3 shows the nucleotide sequence of a complete cDNA clone of OMTI from a stem tobacco library.

10   Figure 4 is the nucleotide sequence of a OMT III cDNA isolated from a λZapII library of tobacco leaf.

Figure 5 shows the construction of antisense and sense vectors using the 5' end 500 bp and the
15   3' end 900 bp BamH1 fragments from pPLC4.

Figure 6 shows the construction of an antisense vector using a 1.4 kb PCR fragment containing the complete pPLC4 clone.

The invention will now be described by way of
20   illustration in the following Examples.

EXAMPLE 1

Purification of poplar OMTs

A. Isolation procedure

Table 1 shows the OMT activity using caffeic
25   acid as a substrate in young leaves and xylem tissue from poplar trees (Populus trichocarpa x P. deltoides).

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TABLE 1

<u>OMT Activity in young leaves and</u>
<u>xylem tissue from poplar trees</u>

Tissue	Activity/g tissue n kat/g	Specific Activity n kat/g protein
Leaves	0.015	0.002
Xylem	0.6	0.2

There is approximately 50-fold more caffeic
acid O- methyltransferase activity in xylem as
compared to leaves. Subsequently, OMT activity was
purified both from leaves and xylem tissue. The

5   procedure for the purification of the OMTs was
established by Dumas et al., (1988). The
purification of OMT activity from a total protein
extract of poplar leaves via ammonium sulphate
precipitation, desalting on Sephadex G25, Q-

10   Sepharose chromatography, and adenosine agarose
affinity chromatography, and finally, MonoQ
chromatography resulted in one 38-kDa protein.
About 5 μg of OMT was obtained from 100 g of
leaves. Using xylem as source, several

15   purification steps were omitted. Crude protein
extract from 5 g of xylem was applied immediately
on an agarose adenosine column leading to the
purification of about 50 μg of the 38- kDa OMT with
a yield of 50% . At this stage a minor

20   contaminating band of 37-kDa is still visible.
<u>B. Immunological characterisation of poplar OMTs.</u>
        Antibodies raised against OMT I and OMT II
from tobacco were used to test for cross-reactions

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with the poplar OMTs. Antibodies raised against
OMT II cross-react with the proteins (Mr 38 kDa and
70 kDa) on protein gel blots of proteins purified
from leaves by three purification steps (G25,

5   Q-Sepharose, and adenosine agarose affinity
chromatography). Antibodies raised against OMT I
show weak cross- reaction with a 37-kDa protein. A
fourth purification step (chromatography on MonoQ
column) resulted in one 38-kDa protein that

10   cross-reacts solely with antibodies directed
against OMT II on protein gel blots.

C. Inhibition in vitro of the poplar OMT caffeic
acid O-methyltranferase activity by antibodies
against OMT II and OMT I from tobacco.

15   Although the poplar OMT and the tobacco OMT
II have a different substrate specificity, the
protein gel blots clearly indicate that both
enzymes must have similar epitopes recognised by
the rabbit antiserum against tobacco OMT II.

20   Five ng of purified poplar OMTI was mixed
with corresponding amounts of antibodies directed
against tobacco OMTI and OMTII. After incubation
at 37°C, OMT activity towards caffeic acid was
measured. The results are given in Table 2 which

25   shows that the caffeic acid OMT activity of the
purified poplar OMT can be inhibited by adding
antibodies directed against OMT II from tobacco
prior to the OMT activity assay (see Materials and
Methods). Two and four μl of undiluted rabbit

30   antiserum against tobacco OMT II mixed with
purified poplar OMT resulted in a 87% and 92%
inhibition, respectively, of the OMT activity.
However, 2 μl of undiluted anti-OMT I rabbit
antiserum mixed with purified poplar OMT led to a

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29% inhibition of the OMT activity. No inhibition
of the OMT activity was observed by mixing 2 μl of
pre-immune serum with the purified poplar OMT prior
to the OMT activity assay.

TABLE 2

Inhibition in vitro of the poplar OMT
activity by antibodies directed
against OMTI and OMTII from tobacco

Antibody	OMT activity toward Caffeic acid (%)	Inhibition (%)
-	100	0
2μl anti-OMTI	71	29
2μl anti-OMTII	13	87
4μl anti-OMTII	8	92
2μl pre-immune serum	100	0

5   D. Specific activity of the purified poplar OMT
towards different phenolic compounds.

The specific activity of the purified OMT
towards three different O-diphenolic substrates was
measured. Using catechol, caffeic acid, and

10   hydroxyferulic acid as substrate, we found an OMT
activity of 0, 30, and 15 nkat/mg protein,
respectively.

Previously, it has been shown that OMT 1 from
tobacco uses mainly caffeic acid and hydroxyferulic

15   acid as a substrate (Collendavelloo et al., 1981).
Therefore, the purified poplar OMT has an OMT
I-like enzymatic activity.

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EXAMPLE 2

Microsequencing of the 38-kDa OMT.

The 38-kDa protein isolated from poplar xylem
tissue was digested with trypsin and the peptides

5   were separated on reverse-phase HPLC. Four peptides
were sequenced:

peptide 45 (R/KDLPHVIEDAPSYGVEHVGGDMF)

peptide 49 (LVDVGGGTGAVV)

peptide 51 (GINFDLPHVIEDAP)

10   peptide 52 (VILVE?ILPVAPD).

Note that peptide 45 is a mixture of two
peptides with arginine and lysine, respectively, as
first amino acid. Trypsin cleaves proteins after a
lysine or arginine residue, except when this is

15   followed by proline, glutamic acid, or aspartic
acid. This implies that the first amino acid
preceding the sequence of peptide 45 has to be
either an arginine or a lysine. The sequences of
peptide 45 and 51 are overlapping.

20   EXAMPLE 3

Design of oligos and cloning of OMT sequences.

Two amino acid sequences of peptide 49
(VDVGGGTGA) and peptide 51 (PHVIEDAP) were chosen
to design degenerated oligonucleotides which were

25   subsequently used as primers for a PCR with DNA
prepared from a leaf cDNA library of poplar. Since
the relative position of the peptide in the protein
are unknown, both sense and antisense
oligonucleotides were designed. Following the PCR,

30   a 108-bp fragment was found to be amplified with
sense primer 49 and antisense primer 51. Using
both total RNA from xylem and genomic DNA of
poplar, as a template, the same fragment was
amplified. The PCR product was subcloned as a

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blunt-end fragment in the SmaI site of pGem2
yielding plasmid pPLC1. The nucleotide and deduced
amino acid sequences are shown in Figure 1
(positions 661-768).

5　　　　To isolate a full-length cDNA, the 108-bp
fragment was used as a probe to screen a leaf cDNA
library from poplar. Three different clones,
designated pPLC2, pPLC3, and pPLC4 were identified
out of 80,000 clones. The nucleotide sequence of

10　pPLC4 and the deduced amino acid sequence are shown
in Figure 1. The cDNA insert is 1375 nucleotides
in length and contains one open reading-frame of
1092 nucleotides encoding a protein of 364 amino
acids (calculated M 39,720; Pi 5.45). The pPCL2

15　clone contains a cDNA of 1,420 nucleotides and
contains also one ORF of 1,092 nucleotides encoding
a protein of 364 amino acids. There is only a
3-amino acid difference between the proteins of the
pPCL4 and the pPCL2 clones. Amino acids 97, 191,

20　and 361 in the protein of pPCL2 are leucine,
isoleucine, and phenylalanine, respectively. Thus a
mixture of at least three closely related isoforms,
as shown by the two isoforms present in peptide 45
and by the amino acid sequence of peptide 51 which

25　is entirely found back in the deduced OMT sequence.
EXAMPLE 4
Analysis of the transgenic poplar trees for a
modified lignin content
A.　　Transformation of poplar

30　　　　With the poplar OMT cDNA clone (pPCL4) five
different sense and antisense constructs were made
resulting in five plasmids: p35SSOM3A (a 500 bp
5'end fragment in sense), p35SASOM3A (a 500 bp 5'
end fragment in antisense), p35SSOM3B (a 900 pb 3'

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end fragment in sense), p35SASOM3B (a 900 pb 3' end
fragment in antisense) and p35SASOM3C (the full
length OMT clone in antisense). All the constructs
are under the control of the constitutive 35S

5 Cauliflower Mosaic Virus (CaMV) promoter. These
constructs were introduced in poplar via an
'Agrobacterium tumefaciens' – mediated
transformation of stem explants.

   For the plasmids p35SSOM3A (SA), p35SASOM3A

10 (ASA), p35SSOM3B (SB) and p35SASOM3B (ASB) 16,21,7
and 13 independent transformants were regenerated,
respectively. Control transformations were
performed using plasmid pGSJ780A (this plasmid
contains only the 35S CaMV promoter).

15   2.   Estimation of the OMT antisense RNA
amount in leaves from ASA and ASB plants.

   Using single stranded (ribo) probes (which
only hybridise with the antisense RNA) RNA gel
blots were performed. The antisense B RNA was

20 detected (strongly) in candidate ASB 5B and
candidates ASB 3Am ASB 5A and ASB 7A.

   The antisense A RNA levels were high in
candidates ASA 1A and candidates ASA 5B, ASA 17A,
ASA 6A and ASA 2B. No antisense RNA was detected

25 in transgenic plants containing the pGSJ780A T-DNA.
The difference in the antisense RNA levels can be
explained by position effects.

3.   Steady-state OMT sense RNA in leaves from SA
and SB plants.

30   In an analogous way, by performing Northern
blots with single stranded riboprobes which only
detect sense RNA, SA and SB plants were analysed.

   The sense B RNA can be detected easily in
candidates SB 5A and SB 8A and not in candidates SB

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2B, SB 4A, SB 7A and SB 10A.

The sense A RNA amount is high in candidates
SA 11A and SA 12A, low in candidates SA 9A, SA 18A
and SA 23A.

5   In all the transformants the endogenous OMT
mRNA could not be detected (this is in agreement
with the fact that the OMT is strongly expressed in
xylem and very weak in leaves). All these
different transgenics have the same phenotype as

10   the wild type poplar and control plants.

4. OMT activity towards caffeic acid in the
transgenic plants.

The OMT activity was measured in different
organelles/tissues (Tables 3 and 4). Two

15   candidates (p35SASOM3B 4A and 6A) with a lower OMT
activity in petioles, xylem and phloem (two to
three times lower) in comparison to wild type and
control plants, were identified.

For both these candidates, there was no

20   correlation between the reduced OMT activity and
the amount of antisense RNA present in these
plants.

5. Lignin analysis of the transgenic poplars.

The transgenic plants were analysed for their

25   lignin composition (Table 5). Lignin is a complex
polymer of three different units: p-hydroxyphenyl
(H), guaiacyl (G) and syringyl (S) monomers.
Poplar lignin contains both guaiacyl and syringyl
units. A typical syringyl/guaiacyl (S/G) ratio for

30   poplar is 2/1. Due to the antisense inhibition we
expect that the monomer ratio will be modified,
resulting in a lower S/G ratio. Lignin
characterisation of xylem was performed by the
analysis of degradation products recovered from

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thioacidolysis. This method allows the estimation
of the different units, involved in the lignin
characteristic structure.

5　　　　For the wild type and control plants a typical
S/G ratio was found (2/1, Table 5). However, the
two candidates ASB 4A and ASB 6A have a lower S/G
ratio, 1.47 and 1.43, respectively. Like the bm3
maize mutants (these mutants have a lower S/G ratio
(.039) in comparison with wild type maize (1.72) we
10　　notice that the S/G ratio of the two ASB poplars
has been modified (although not as drastically as
was the case for the maize mutants).

TABLE 3
OMT activity in petioles of the different ASB, ASA,
SB and SA plants (in cpm).

Sample		Replicates			Mean
		1	2	3	
ASB	6A	–	2460	1252	1856
	8A	6074	5710	6931	6238
	4A	1097	1946	711	1251
	5B	2419	6295	5764	4826
ASA	23A	1954	4342	3834	3376
	17A	5001	7596	5386	5994
	2B	2602	3926	5796	4108
SB	4A	2527	5799	5226	4517
	10A	2252	4970	5631	4284
	5A	4726	4672	4838	4755
	7A	2686	6302	7490	5492
SA	13A	3638	5423	3697	4252
	3B	3269	4201	6875	4781
	2B	2479	6626	5842	4982
	7A	5338	8244	8691	7424
WT	T1	3231	2843	7577	4550
	T2	3996	6171	6433	5533
	T3	3470	5152	10028	6216

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WT = Control Plants.

TABLE 4

<u>OMT activity in xylem and phloem of the candidates</u>
<u>ASB 4A and ASB 6A (in cpm). Mean value, last lane.</u>

Sample		Replicates 1	2	3	Mean
ASB	4AX F	2465 3530	1624 1108	743 1482	1610 2040
ASB	6AX F	3089 4258	1845 2063	1152 3143	2028 3154
WT	T1X F	5040 8665	4440 5505	2262 8809	3914 7659

WT = Control plants

TABLE 5

<u>Syringyl/guaiacyl ratio of the different ASB, ASA,</u>
<u>SB and SA plants.</u>

Sample		Replicates 1	2	Mean
ASB	18A 5A 4A 6A	2.17 1.61 1.34 1.28	2.41 1.84 1.60 1.59	2.29 1.73 1.47 1.43
ASA	7B 15A 23A	1.93 1.80 1.88	2.48 2.35 2.13	2.20 2.07 2.00
SB	4A	1.95	2.09	2.02
SA	1B	2.07	2.20	2.14
WT	T1	2.02	1.98	2.00

WT = Control Plants

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EXAMPLE 5

<u>Isolation of a tobacco OMT1 clones pTOMT1A and</u>
<u>pTOMT1B.</u>

Using similar procedures described for the
5   isolation of the poplar OMT cDNA, a tobacco OMT1
cDNA clones were isolated from a leaf cDNA library
prepared from RNA of TMV infected tobacco leaves.
These clones were isolated by PCR using sequence
information of the purified tobacco OMT1 protein.
10   The combined sequence of two clones covering the
complete coding sequence of tobacco OMT1 are shown
in Figure 2. The underlined part represent
sequences found in clone A. the dotted part
represents sequences found in clone B.
15   A complete cDNA clone of OMTI has been
isolated from a stem cDNA tobacco library. Its
sequence is shown in Figure 3.

EXAMPLE 6

<u>Immunoscreening of a λgt11 expression library and</u>
20   <u>characterisation of the MOT III cDNA clone o3.614</u>

The λgt11 phages were plated on Escherichia
coli Y1090 and fusion proteins induced with 10mM
IPTG. The proteins bound to nitro-cellulose
filters were screened by immunodetection using
25   anti-tobacco OMT III polyclonal antibody (Hermann
et al., 1987; Dumas, 1990). Three positive clones
were purified after immunoscreening of the λgt11
library. The length of their cDNA inserts was
determined by PCR. Aliquots of phage lysates (5μl)
30   were heated for 5 mins at 94°C and used as template
for PCR. The amplification mixture consists of
10mM Tri-HCl pH 8.3, 11mM KCl, 1.6mM $MgCl_2$, 1mM
DTT, 200μM each dNTP, 1μM each oligonucleotide
primer, and 1 unit of Taq polymerase (Beckman) in a

- 25 -

final volume of 50µl. The amplification program
consisted of 25 cycles of denaturation (94°C, 1
min), annealing (40°C, 1 min), and primer extension
(72°C, 1 min). The longest clone, named o3.614,

5   was 614 bp long and was submitted to phage
amplification and DNA purification by CsCl gradient
(4). The 614 bp cDNA insert was subloned by EcoRI
restriction digestion in pBluescript
KS(+)(Stratagene, Inc). According to standard

10   methods (4), deletion from both the extremities of
the plasmid were generated by ExoIII-Mung Bean
digestions and the primers and the T7 DNA
polymerase.

EXAMPLE 7

15   <u>Construction and screening of a λZapII cDNA
library from tobacco leaf RNA and characterisation
of a complete clone</u>

Poly(A)+RNA from 48 hours TMV infected leaves
was used to construct an oligo(dT) primed cDNA.

20   Double-stranded cDNA was ligated to
hemi-phosphorylated EcoR I/Not I adaptors
(Pharmacia), ligated into λZapII vector
(Stratagene, Inc) and packaged using Gigapack in
vitro packaging extracts (Stratagene, Inc). The

25   resulting cDNA library titled 1.8×10^7 pfu/µg
cDNA. The o3.614 clone was used as DNA probe. The
614 bp DNA fragment was purified from purified from
0.8% agarose gel (PrepAGene, BIORAD), 32p labelled
by random oligonucleotide-primed synthesis and used

30   to screen a λZapII library made by standard
protocols (4). After three cycles of screening,
twelve positive clones were isolated from
approximately 1.8×10^5 plaque-forming units. cDNA
inserts from positive phage were rescued as

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Bluescript plasmids by R408 helper phage mediated
in vivo excision, as described by the manufacture
(Stratagene, Inc). Cloned insert DNA was isolated
by Not I digestion and analysed on 1% agarose gels.

5   The cDNA clones isolated from λZapII cDNA library
were mapped with restriction endonucleases and then
sequenced on both strands by the dideoxy chain
termination method 95) using T7 DNA polymerase
(Pharmacia). For sequencing the internal regions,

10   synthetic oligonucleotide primers (18mers) were
designed from the DNA sequence previously
determined. The complete sequence of a OMT III
cDNA clone is given in Figure 4. The amino acid
sequences of peptides obtained by trypsic

15   digestion of the purified OMT II and OMT III
proteins all correspond to the aminoacid sequence
derived from OMT III cDNA clones. These evidences
suggest the occurrence of only one mRNA species for
the "Pathogenesis-Related" forms OMT II and OMT III

20   but of 2 forms for the lignification-related OMT I.
EXAMPLE 8
Localisation of OMT I transcripts by in situ
hybridisation.
        In petiole sections, OMT I mRNAs were

25   localised in parenchyma cells of xylem and phloem,
with a marked signal around the nuclei. In leaf
sections, OMT I mRNAs were found to accumulate
particularly in the upper and lower epidermis in a
ring of tissue surrounding TMV-induced necrotic

30   lesions, were no cell-type specific hybridisation
was found in the healthy leaf.
EXAMPLE 9
Design of antisense vectors
        Five different poplar OMT antisense constructs

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have been made. A 500 bp BamHI fragment
corresponding to the 5' end of the poplar OMT clone
and a 900-bp BamHI fragment corresponding to the 3'
end of the poplar OMT clone were cloned in the
5   BamHI site of vector pGSJ780A, in both sense and
antisense direction yielding the four plasmids
p35SSOM3A, p35SASOM3A, p35SASOM3A, p35SSOM3B, and
p35SASOM3B (Figure 5). All OMT fragments are under
the control of the CaMV 35S promoter. The
10   full-length OMT clone was cloned in the vector
pGSJ780A in antisense direction by PCR yielding
plasmid p35SASOM3C (Figure 6). The correct
direction of the inserts has been confirmed by
sequencing. The vector pGSJ780A is a binary vector
15   with the pVS1 origin of replication and a Sm/Sp
resistance gene for selection in Agrobacterium
tumefaciens. Between the T-DNA borders there is a
nos-nptII-ocs cassette and a multiple cloning
site).

20   The construction of the tobacco antisense
vector follows that described for the poplar
vectors. The insert was inserted into the vector
pGSJ780A.

CLAIMS

1.   A DNA encoding caffeic acid O-methyl
transferase contained in the clones pPLC4 and
pTOMTI and variants thereof such as are
permitted by the degeneracy of the genetic
5   code or the functional equivalents thereof.

2.   A recombinant DNA comprising the DNA
claimed in claim 1 under control of a
transcriptional control sequence operative in
plants.

3.   A recombinant DNA as claimed in claim 2,
for the down-regulation of lignin biosynthesis
in which the said DNA is in antisense
orientation.

4.   A recombinant DNA as claimed in claim 2,
for the amplification of lignin biosynthesis,
in which the said DNA is in sense orientation.

5.   A DNA comprising a transcriptional
initiation region operative in plants
operatively linked to a DNA sequence encoding
RNA complementary to a substantial number of
5   bases showing substantial homology to an mRNA
encoding the protein produced by the gene in
pPLC4 or pTOMT1 so as to initiate production
of mRNA therefrom.

6.   A DNA having the nucleotide sequence given in Figure 1.

7.   A DNA having the nucleotide sequence given in Figure 2.

8.   A DNA having the nucleotide sequence given in Figure 3.

9.   A DNA having the nucleotide sequence given in Figure 4.

10.   A plant cell, and a plant derived therefrom having stably incorporated in its genome by transformation a DNA as claimed in any one of claims 1 to 9 in sense or antisense
5   orientation, and the fruit and seeds of said plants.

11.   A method for the regulation of lignin biosynthesis in a plant comprising stably incorporating into the genome of a plant by transformation a recombinant DNA encoding the
5   enzyme caffeic acid O-methyl-transferase in sense or antisense orientation under control of a transcriptional initiation region operative in plants.

FIG.1

FIG. 2(1/2)

```
1    GCCGGCCCGG GCGCGGCGAT TTCTCCTTCT GAATTAGCTG CTCAGCTCTC

51   AACCCAGAAC CCAGAAGCAC CCGTTATGCT TGATCGGATG CTTAGGCTAC

101  TTGCTACTTA CTCTGTTCTC AATTGTACTC TTAGAACACT GTCTGATGGC

151  AGTGTTGAGA GGCTTTATAG TCTGGCTCCG GTTTGTAAGT TCTTGACTAA

201  GAATGCTGAT GGTGTTTCTG TTGCCCCACT TTTGCTTATG AATCAAGATA

251  AAGTTCTTAT GGAGAGCTGG TACCACTTAA AAGATGCAGT ACTAGATGGT

301  GGAATCCCAT TCAACAAGGC CTATGGAATG ACAGCATTTG AGTACCATGG

351  CACAGATCCA AGATTCAACA AAGTTTTCAA CCGTGGAATG TCTGATCACT

401  CCACTATGTC AATGAAAAAG ATTCTTGAGG ACTACAAAGG ATTTGAAGGC

451  CTAAATTCCA TTGTCGATGT TGGTGGTGGA ACTGGCGCTA CTGTTAACAT

501  GATTGTCTCC AAACATCCCT CTATTAAGGG TATTAACTTT GATTACCAC

551  ATGTTATTGG AGATGCTCCA GCTTCACCTG GTGTCGAGCA CGTTGGTGGC

601  GACATGTTTG CCAGTGTGCC AAAAGCAGAT GCCATTTCA TGAAGTGGAT
```

```
 651  TTGTCATGAT TGGAGCGACG AGCATTGCCT AAAATTCTTG AAGAATTGCT
 701  ATGAAGCACT ACCTGCAAAT GGGAAGGTGA TAATAGCGGA GTGCATACTT
 751  CCAGAGGCCC CAGATACATC ACTTGCAACT AAGAATACAG TACATGTTGA
 801  TATTGTGATG TTAGCACATA ACCCAGGAGG CAAAGAAAGG ACTGAGAAGG
 851  AATTTGAGGC TTTGGCTAAG GGCGCTGGTT TTACTGGATT CGCAAGGCTT
 901  GTTGCGCTTA CAACACTTGG GTCATGGAAT TCAACAAATA ATTAATCGAT
 951  TCCTTTGGAG AATTAAGCAA TATACTGTTC ATTTGCATT TTGAAATTCT
1001  ACTTTTCACA GAGTGGGCTTT ACTGCGAAAT AAAAGAAATA TATAGCTTTT
1051  ACCTTGAAAA GATCAATGTT CAAAGGGAAA AAAAAAAGGA AGATGAAATA
1101  ATTGCTCTCA GAAAAGCAGT GTGTTAGGAA AAAGCTTTT AGCTGGATTT
1151  TGAATTTTTA TTGTATGTAT TTCTGTAATA CACATGTATT GAAGGAATAC
1201  TAGTTTTCGA CCAATCATAT TTCTTTGAAA AAAAAAAAAA AAAA
```

FIG.2(2/2)　OMT I Sequences : plasmid A + plasmid B

——————— : A

------------- : B

FIG. 3 (1/2)

```
ATTCCTTCAACTTACCCAATTAAGTCATCGAAAAATCTGAAACAGAACTAAAAGTAAAAT
1
                                                             M

TTATTTGCCATGCAATTGTGTAGTGCTTCTGTACTTCCTATGGTCCTAAAATCAGCCGTAGA
119
 L  F  A  M  Q  L  C  S  A  S  V  L  P  M  V  L  K  S  A  V  E
21

GAATTAGCTGCTCAGCTCTCAACTCAGAACCCAGAAGCACCTGTTATGCTTGATCGGATGCT
239
 E  L  A  A  Q  L  S  T  Q  N  P  E  A  P  V  M  L  D  R  M  L
61

AGTGTTGAGAGGCTTTATAGTCTGGCTCCCGTCTGTAAGTACTTGACTAAGAATGCTGATGG
359
 S  V  E  R  L  Y  S  L  A  P  V  C  K  Y  L  T  K  N  A  D  G
101

TACCACTTAAAAGATGCAGTACTAGATGGCGGAATCCCATTCAACAAAGCCTATGGAATGCA
419
 Y  H  L  K  D  A  V  L  D  G  G  I  P  F  N  K  A  Y  G  M  T
141

TCTGATCACTCCACTATGTCAATGAAGAAGATTCTTGAGGACTACAAAGGATTTGAAGGCCT
599
 S  D  H  S  T  M  S  M  K  K  I  L  E  D  Y  K  G  F  E  G  L
181

AAATATCCCTCTATTAAGGGCATTAACTTTGATTTGCCACATGTAATTGGAGATGCTCCAAC
719
 K  Y  P  S  I  K  G  I  N  F  D  L  P  H  V  I  G  D  A  P  T
221

GCCATTTTCATGAAGTGGATTTGTCATGATTGGAGCGATGAGCATTGCCTAAAATTCTTGAA
839
 A  I  F  M  K  W  I  C  H  D  W  S  D  E  H  C  L  K  F  L  K
261

CCAGAGGCCCCAGATACATCACTTGCAACTAAGAATACAGTACATGTTGATATTGTTATGTT
959
 P  E  A  P  D  T  S  L  A  T  K  N  T  V  H  V  D  I  V  M  L
301

GGCGCTGGTTTTACTGGATTCGCAAGGCTTGTTGCGCTTACAACACTTGGGTCATGGAATTC
179
 G  A  G  F  T  G  F  A  R  L  V  A  L  T  T  L  G  S  W  N  S
341

CATTTGGAAATTCTACTTTTCTCAGAGTGGCTTGACTGTGAAATAAAAGAAATATAGCTTTT
1199

AAAGCAATGTGTTAGGAAAAGCTTTTTTAGCTGGATTTTGAATTTTACTGTATGTATTTCT
1319
```

FIG. 3 (2/2)

```
GGGTTCAACAAGCGAGAGCCAGAGTAACAGTCTCACTCACACAGAAGACGAAGCTTTC
                                                         118
  G  S  T  S  E  S  Q  S  N  S  L  T  H  T  E  D  E  A  F
                                                          20
ACTTGACCTTCTTGAGCTAATGGCTAAGGCTGGTCCAGGTGCAGCTATTTCTCCTTCT
                                                         238
  L  D  L  L  E  L  M  A  K  A  G  P  G  A  A  I  S  P  S
                                                          60
TAGGCTACTTGCTTCTTACTCTGTTCTCAATTGTACTCTTAGAACACTGCCTGATAGC
                                                         358
  R  L  L  A  S  Y  S  V  L  N  C  T  L  R  T  L  P  D  S
                                                         100
TGTTTCTGTTGCCCCACTTTTGCTTATGAATCAAGATAAAGTTCTTATGGAGAGCTGG
                                                         418
  V  S  V  A  P  L  L  L  M  N  Q  D  K  V  L  M  E  S  W
                                                         140
AGCATTTGAGTACCATGGCACAGATCCAAGATTCAACAAAGTGTTCAACCGTGGAATG
                                                         598
  A  F  E  Y  H  G  T  D  P  R  F  N  K  V  F  N  R  G  M
                                                         180
AAATTCCATTGTTGATGTTGGTGGTGGAACGGGTGCTACTGTTAACATGATTGTCTCT
                                                         718
  N  S  I  V  D  V  G  G  G  T  G  A  T  V  N  M  I  V  S
                                                         220
TTACCCCGGTGTCGAGCACGTTGGTGGCGACATGTTTGCTAGTGTGCCAAAAGCAGAT
                                                         838
  Y  P  G  V  E  H  V  G  G  D  M  F  A  S  V  P  K  A  D
                                                         260
GAATTGCTATGAAGCACTACCTGCAAATGGGAAGGTGATAATTGCAGAGTGCATACTT
                                                         958
  N  C  Y  E  A  L  P  A  N  G  K  V  I  I  A  E  C  I  L
                                                         300
AGCACATAACCCAGGAGGCAAAGAAAGGACTGAGAAGGAATTTGAGGCTTTGGCTAAG
                                                        1078
  A  H  N  P  G  G  K  E  R  T  E  K  E  F  E  A  L  A  K
                                                         340
AACAAGTAATTAATCGATTCCTTAATTTGAAGGATTAAGCAATATACTGTTCGTTTTG
                                                        1198
  T  S  N  *
                                                         364
AACTTGAAAAGATTGATGTTCAAAAGAAAAAAGGAAGATGAAATAATTGCTCTCAGA
                                                        1318
GTTATACACATGTATTGAAGGAATACTAGTTTTCGACCAAAAAAAAAAAAAA
                                                        1431
```

FIG.4(1/5) OMT III cDNA isolated from the λ Zap II library of tobacco leaf

```
  1  AACAAAAACACTCTAAAAGGAAAAGACTAGGAGGAGAATAATTACACTACAACTCTTATAGCT

 61  AATTCTTGTCTCAAGATTTTCACCTATGGAATCCTCAACCAAAAGCCAAATACCAACACA
                              M  E  S  S  T  K  S  Q  I  P  T  Q   12

121  ATCAGAAGAAGAGAGCCGTAACTGCACATATGCCATGCAACTATTGTCATCTTCAGTCCTCCC
      S  E  E  R  N  C  T  Y  A  M  Q  L  L  S  S  S  V  L  P   32

181  CTTTGTGTTGCATTCAACAATTCAATTGGAAGTTTTTGAGATATTAGCCAAATCTAATGA
      F  V  L  H  S  T  I  Q  L  E  V  F  E  I  L  A  K  S  N  D   52

241  CACTAAACTTTCTGCTTCTCAAATTGTTTCTCAAATTCCTAACTGCAAGAATCCCTGATGC
      T  K  L  S  A  S  Q  I  V  S  Q  I  P  N  C  K  N  P  D  A   72
```

FIG. 4 (2/5)

```
301  AGCTACTATGTTAGATAGGATGCTTTATGTGTTGGCTAGTTACTCGTTGTTACTTGTTC          92
      A  T  M  L  D  R  M  L  Y  V  L  A  S  Y  S  L  F  T  C  S

361  CATTGTTGAGGATGAAGAAAATAATGGGGGCCAGAAAAGAGTGTATGGTTTGTCACAAGT      112
      I  V  E  D  E  E  N  N  G  G  Q  K  R  V  Y  G  L  S  Q  V

421  GGGAAAATTCTTGTTAGAGATGAAGATGGTGCATCAATGGGGCCACTTTTGGCTTTGCT      132
      G  K  F  F  V  R  D  E  D  G  A  S  M  G  P  L  L  A  L  L

481  TCAAGATAAAGTATTCATAAACAGCTGGTTTGAACTAAAGAGATGCAGTTCTTGAAGGAGG    152
      Q  D  K  V  F  I  N  S  W  F  E  L  K  D  A  V  L  E  G  G

541  AGTTCCATTTGACAGGGTACACGGTGTTGTCCATGCATTGAATATCCAAAATCGGACCC      172
      V  P  F  D  R  V  H  G  V  V  H  A  F  E  Y  P  K  S  D  P
```

FIG. 4 (3/5)

```
601  AAAATTCAATGATGTTTCAACAAGGCAATCAATCACACAACTGTAGTCATGAAAAA
         K  F  N  D  V  F  N  K  A  M  I  N  H  T  T  V  V  M  K  K  192

661  AATACTTGAAAATTACAAAGGTTTTGAGAACCTTAAAACTTTGGTTGATGTTGGAGGTGG
         I  L  E  N  Y  K  G  F  E  N  L  K  T  L  V  D  V  G  G  G  212

721  TCTTGGGAGTTAACCTCAAGATGATTACATCTAAATACCCCACAATTAAGGGCACTAATTT
         L  G  V  N  L  K  M  I  T  S  K  Y  P  T  I  K  G  T  N  F  232

781  TGATTGCCACATGTTGTTCAACATGCCCCTTCCTATCCTGGGGGTGGAACATGTTGGGGG
         D  L  P  H  V  V  Q  H  A  P  S  Y  P  G  V  E  H  V  G  G  252

841  AGATATGTTTGAAAGTGTTCCAGAAGGAGATGCTATTTTTATGAAGTGGATTCTTCATGA
         D  M  P  F  S  V  P  E  G  D  A  I  F  M  K  W  I  L  H  D  272
```

FIG.4 (4/5)

```
 901 CTGGAGTGATAGTCACAACCTCAAGTTGCTAAAGAACTGCTACAAGGCTCTACCAGACAA
        W  S  D  S  H  N  L  K  L  L  K  N  C  Y  K  A  L  P  D  N   292

 961 TGGAAGGTGATTGTTGTTGAGGCCATTTTACCAGTGAAACCAGACATTGACACCGCAGT
        G  K  V  I  V  V  E  A  I  L  P  V  K  P  D  I  D  T  A  V   312

1021 GGTTGGCGTTTCGCAATGTGATTTGATCATGATGGCTCAAAATCCTGGAGGCAAAGAGCG
        V  G  V  S  Q  C  D  L  I  M  M  A  Q  N  P  G  G  K  E  R   332

1080 ATCGGAAGAGGAGTTTCGAGCCTTGGCTACTGAAGCTGGATTCAAAGGGCGTTAACTTAAT
        S  E  E  E  F  R  A  L  A  T  E  A  G  F  K  G  V  N  L  I   352

1141 ATGTTGTGTCTGTAATTTTTGGGTCATGGAATTCTGCAAGTAGATTTCTACTGTACATTG
```

```
       C   C   V   C   F   P   W   V   M   E   F   C   K   *

1201   AGTTTCTACTACTCTTGAGTATCCATTTATGGCAATCTGGGACTGGAATTGCAGCTTAGT

1261   CCAGATTGAACATTGATATTCCTAATAATATTTCTATTATTCCCTTGTTTATTTCTCTT

1321   GTATGAAAGGATGTCATTTTGAGTATTGATAATCATGTTCTCTAGGACAGAAATTGTAAC

1381   TTTGTCCAACTTTATTGATATTCCTAGTAAGATTTATATGACATGTGTCTCTGGTTTGAG

1441   AAGAGTTTCAATATCTAAAAAAAAAAAAAA
```
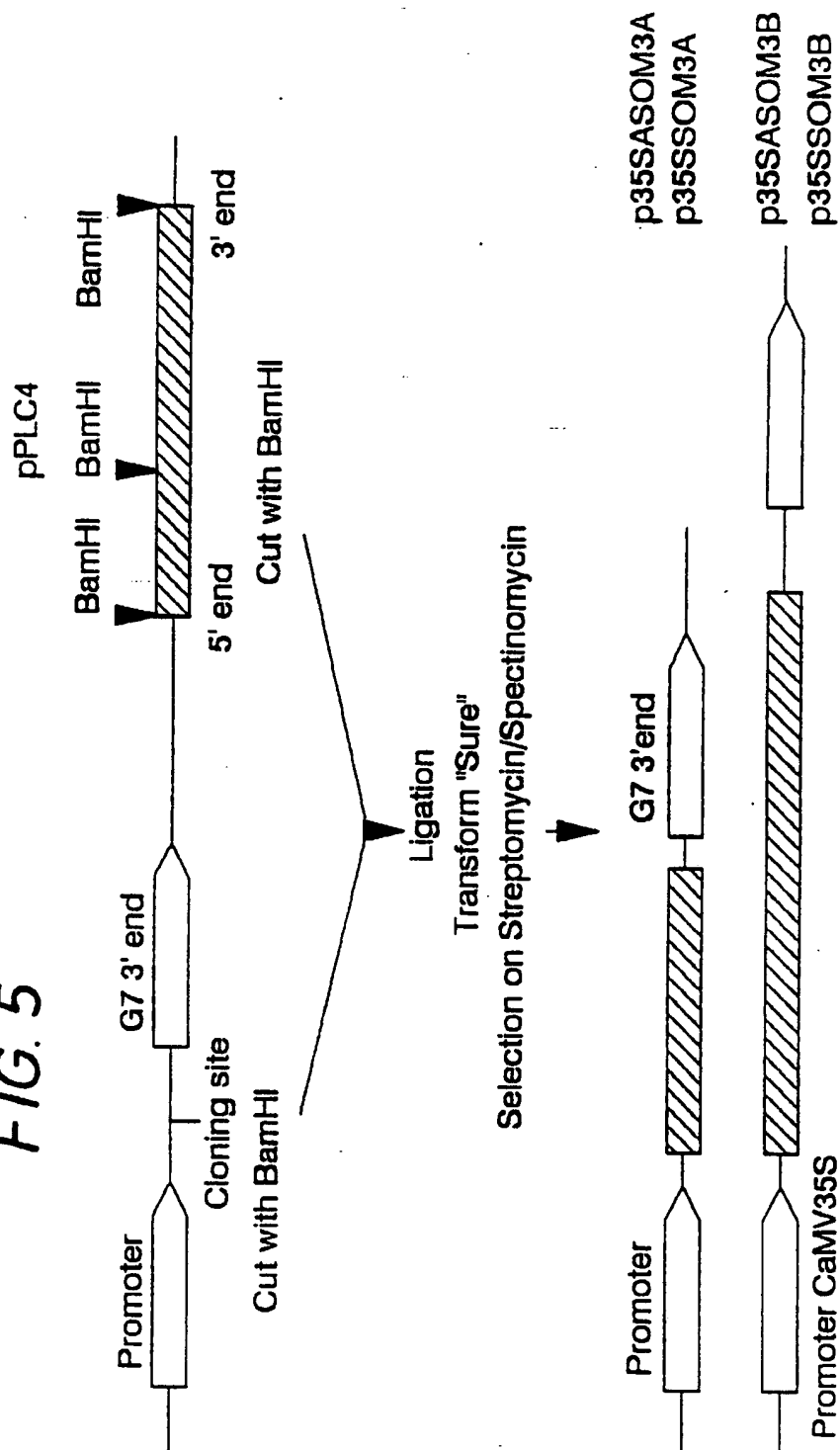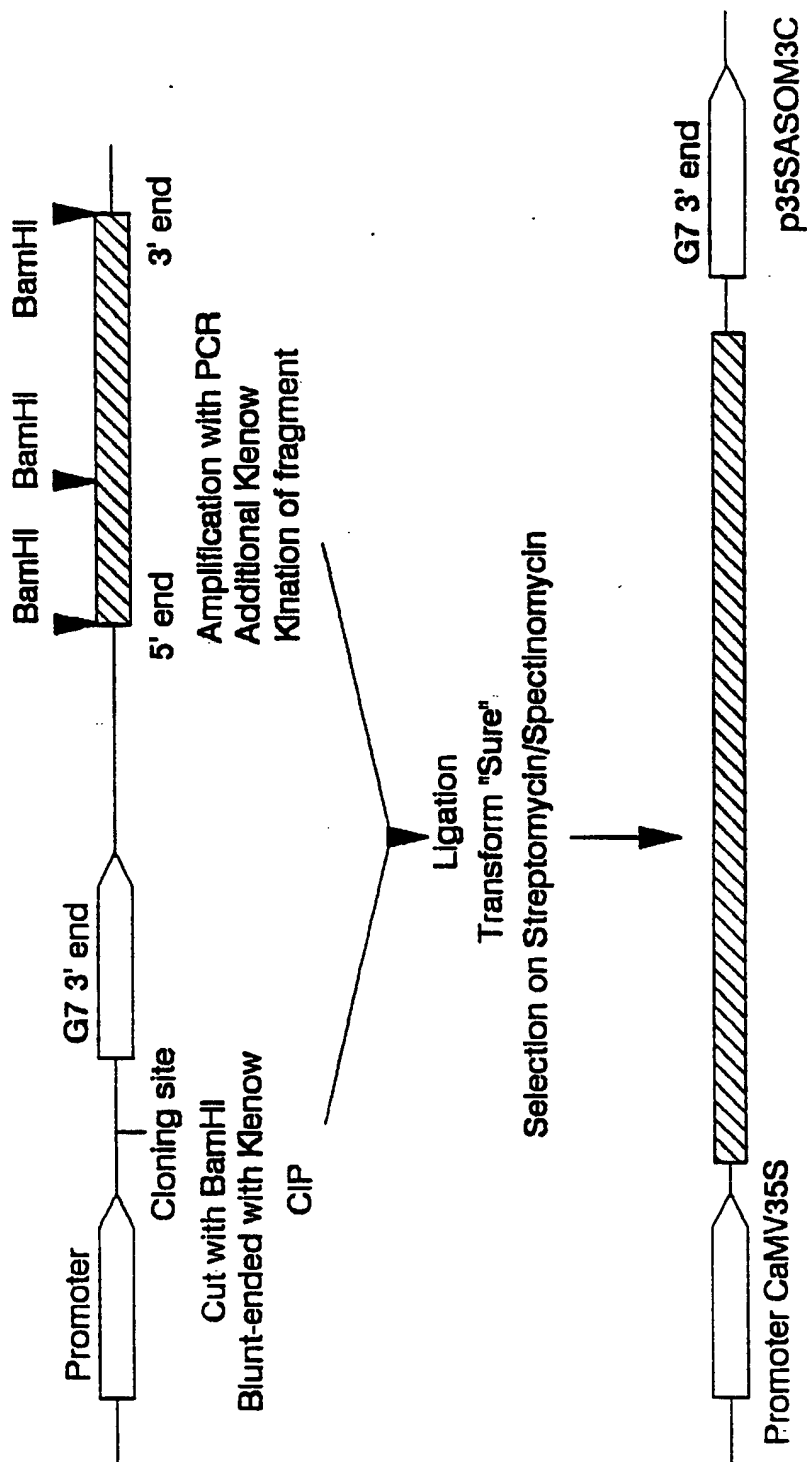
FIG. 4 (5/5)

FIG. 5

Construction of sense and antisense vectors

FIG.6

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]
According to International Patent Classification (IPC) or to both National Classification and IPC
Int.Cl. 5 C12N15/54;   C12N15/82;   C12N15/11;   C12N5/10

II. FIELDS SEARCHED

Minimum Documentation Searched[7]	
Classification System	Classification Symbols
Int.Cl. 5	C12N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[o]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	PLANT PHYSIOLOGY. vol. 97, no. 1, September 1991, ROCKVILLE, MD, USA. pages 7 - 14 GOWRI, G., ET AL. 'Stress responses in Alfalfa (Medicago sativa L.)'	1
Y	see the whole document particularly page 13 right col. last para ---	2-11
X	J. CELL. BIOCHEM. SUPPL.,MEETING HELD JAN 10-17, 1991. vol. 15A, 1991, page 144 CAMPBELL, W.H., ET AL. 'Seasonal expression of a lignin specific O-methyltransferase cloned from aspen developing secondary xylem' see the whole document ---	1
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° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
08 DECEMBER 1992	10. 12. 92
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	MADDOX A.D.

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category°	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	PLANT PHYSIOLOGY, (SUPPL.) vol. 93, no. 1, May 1990, ROCKVILLE, MD, USA. page 15 BUGOS, R.C., ET AL. 'Cloning and expression of lignin bispecific O-methyltransferase' see abstract 83 ---	1
Y	CA,A,2 005 597 (SCHUCH) 15 June 1990 see page 13, line 14 - line 34 ---	2-5,10, 11
Y	BIOLOGICAL ABSTRACTS vol. 73 , 1982, Philadelphia, PA, US; abstract no. 64323, MARIGO, G., ET AL. 'Biosynthesis of carbon-14-labeled ferulic acid and sinapic acid' see abstract & J. LABELLED COMPD. RADIOPHARM. vol. 18, no. 5, 1981, pages 695 - 702 ---	6
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P,X	PLANT PHYSIOLOGY. vol. 98, no. 2, February 1992, ROCKVILLE, MD, USA. pages 796 - 797 DUMAS, B., ET AL. 'Nucleotide sequence of a complementary DNA encoding O-methyltransferase from poplar' see the whole document ---	6

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III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category°	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	BIOLOGICAL ABSTRACTS vol. 94 , 1992, Philadelphia, PA, US; abstract no. 89217, JAECK, E., ET AL. 'Regulation of enzymes involved in lignin biosynthesis: Induction of O-methyltransferase mRNAs during the hypersensitive reaction of tobacco to tobacco mosaic virus' see abstract & MOL PLANT-MICROBE INTERACT. vol. 5, no. 4, 1992, pages 294 - 300 ---	7,8
O,P, X	J. CELL. BIOCHEM. SUPPL., MEETING HELD APRIL 10-16, 1992. vol. 16F, 1992, page 219 NI, W., ET AL. 'Modification of lignin biosynthesis by genetic manipulation of caffeic acid O-methyltransferase' see abstract Y219 ---	3-5,10, 11
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The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CA-A-2005597	15-06-90	None	